BIOCHEMICAL BASIS OF ACIDITY IN CITRUS FRUITS

By
BE'LA STEPHEN BUSLIG

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Béla Stephen Buslig

December, 1970

Chairman: Dr. R. H. Biggs
Co-Chairman: Dr. J. A. Attaway
Major Department: Fruit Crops

The metabolism and accumulation of organic acids in citrus fruits, with emphasis on citric acid, were investigated. The problem was approached by examining the accumulation patterns of titratable organic acids in various fruits, while concurrently studying respiration, both in the entire fruit and in subcellular fractions (mitochondria). The enzymes citrate synthetase, isocitrate dehydrogenases and isocitritase were studied. Results indicated better coordination of enzymic activity and respiration in less acid fruit. Examination of ATP and ADP levels in fruits of varying acidity indicated the involvement of these nucleotides in respiratory control and probably active transport of overproduced citric acid into a separate compartment of the juice sacs, probably the vacuole. The results obtained from CO_2 fixation studies by whole fruit seemed to discount the idea that carboxylation reactions are significant contributors to acid production. Another experiment concerned with the action of arsenate on respiration indicated that arsenate acts in competition with phosphate, and not as an uncoupler.

Experimental evidence also points to greater respiratory efficiency in less acid fruit mitochondria, with better respiratory control by exogenous ADP. Conclusions drawn from the experiments point to coordination of enzymic activities as the main pathways in controlling organic acid accumulation.

INTRODUCTION

The genus Citrus includes the largest single group of economic fruit producing plants in the world. Among the numerous diverse characteristics of these fruits is the remarkable variation of acidity within the group, or even within varieties of a single species. It ranges from 0.06 to 8.00% titratable acids, expressed as citric acid, in the juices of the different variants.

The metabolism and accumulation of organic acids, especially citric acid which predominates in most varieties, is therefore of particular importance. An understanding of the basic processes involved could possibly contribute means to control acidity levels in these fruits. Investigation of the biochemical pathways leading to the synthesis and degradation of citric acid, primarily reactions associated with the Krebs cycle and some of the associated reactions, could provide insight into the regulation of organic acid metabolism. Eventual deciphering of the enzymic mechanisms and their integrated control could possibly be used to devise ways to control acid accumulation, which involves the phenomenon of membrane transport, across both the mitochondrial and the vacuolar membranes. Accumulation involves transport against a concentration gradient. Energy relationships obtained during the growth cycle of the fruit integrated with the enzymological and acidity accumulation data are expected to shed light on the biochemical basis of citrus fruit acidity.

LITERATURE REVIEW*

Changes in organic acids in different citrus fruits were the subject of the first lines of investigation into acid accumulation. Two distinct patterns of accumulation were distinguished.

Lemons (Citrus limon (L.) Burm.) followed a pattern of continuous accumulation during the growth of the fruit. The increase was greatest during the early growth of the fruit. It continued at a slower rate while the fruit was in a logarithmic growth phase, until the end of the cycle. The increase in titratable acidity was continuous until maturity,

*Abbreviations and terms used in the text are given below: ADP (adenosine diphosphate); ATP (adenosine triphosphate); PEP (phosphoenolpyruvate); dimethyl-POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene); PPO (2,5-diphenyl-oxazole); AcSCoA (acetyl-coenzyme A); CoASH (coenzyme A); NAD (nicotinamide-adenine-dinucleotide, oxidized form); NADH (nicotinamide-adenine-dinucleotide, reduced form); NADP (nicotinamide-adenine-dinucleotide phosphate, oxidized form); NADPH (nicotinamide-adenine-dinucleotide phosphate, reduced form); TRIS (tris-(hydroxymethyl)-aminomethane); MOPS (morpholine-propanesulfonic acid); EDTA (ethylene diamine tetraacetic acid); DMSO (dimethyl-sulfoxide); RCR (respiratory control ratio); P/O (respiratory phosphate:oxygen ratio); BSA (bovine serum albumin); PVP-40 (polyvinylpirrolidone, average molecular weight 40,000); ENZYMES: the trivial name is used, they are identified here by the International Union of Biochemistry Enzyme Commission adopted name and number: adenylate kinase (ATP:AMP phosphotransferase) (EC 2.7.4.3); aconitase (citrate (isocitrate)-hydrolyase) (EC4.1.2.3); citrate synthetase (citrate-oxaoacetate-lyase (CoA-acetylating) (EC 4.1.3.7)); citrate lyase (citrate-oxaloacetate-lyase) (EC 4.1.3.6); citrate cleavage enzyme (ATP: citrate oxaloacetate-lyase (CoA-acetylating and ATP dephosphorylating) (EC 4.1.3.8)); NAD-isocitrate dehydrogenase (L_S-isocitrate: NAD oxidoreductase (decarboxylating) (EC 1.1.1.41); NADP-isocitrate dehydrogenase (L_S-isocitrate: NADP oxidoreductase (decarboxylating) (EC 1.1.1.42)); isocitritase (L_S-isocitrate glyoxylate lyase) (EC 4.1.3.1); PEP carboxylase (Orthophosphate:oxaloacetate carboxy-lyase (Phosphorylating) (EC 4.1.1.31)); PEP carboxykinase (ATP:oxaloacetate carboxy-lyase(transphosphorylating) (EC 4.1.1.32)).

with the apparent dilution effect caused by the increasing fruit volume lagging behind the production (Bartholomew, 1923). A similar pattern was observed in a species termed C. acida (probably a lemon) by Sekhara Varma and Ramakrishnan (1956) in their investigation of sources of acid formation in these plants.

In oranges (C. sinensis (L.) Osbeck) the pattern of acidity accumulation was different. There was an initial rapid rise of titratable acids, followed by a long, gradual decline with increasing size, mainly due to the dilution effect of the increased water content of the fruit. At the onset of maturity this decline was hastened by a net loss of total acids (Sinclair and Ramsey, 1944; Bain, 1958; Rasmussen, 1964; Ting and Vines, 1966).

In grapefruit (C. paradisi Macf.) a pattern comparable to that of oranges was observed (Harding and Fisher, 1945; Ting and Vines, 1966).

Generally, it is believed that accumulation of organic acids is caused by overproduction of a specific acid which cannot be efficiently metabolized due to a partial metabolic block or enzymic defect, possibly genetically controlled within the fruit.

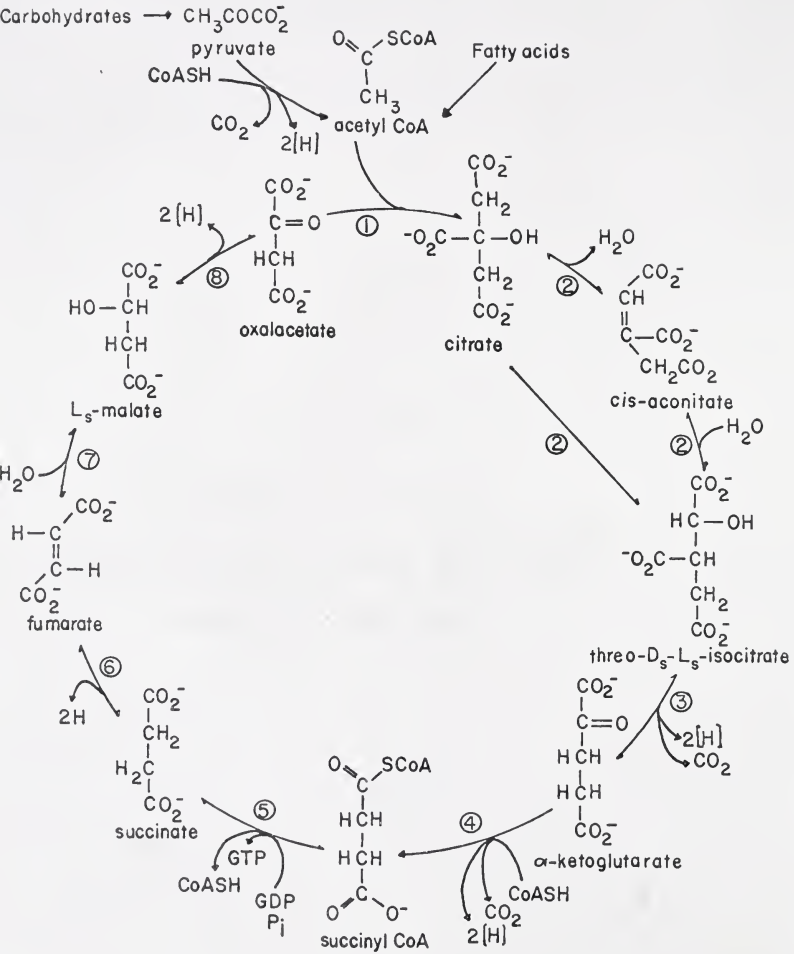
The major organic acid found in citrus fruits, with a few exceptions, is citric acid. In most instances it is present in excess of 70% of the total titrable acidity (Erickson, 1968). In addition to citric acid, malic, succinic, fumaric and quinic acids can be found in appreciable quantities in the juice of the various fruits (Ting and Deszyck, 1959; Clements, 1964 a,b).

All the major acids in the juice, with the exception of quinic, belong to a group which are interconvertiable through a series of reactions known as the tricarboxylic, citric acid or Krebs cycle (Krebs and

Johnson, 1937). Initially, the reactions of the Krebs cycle were deduced from work with animal tissues. Later the enzymes and reactions involved were found to exist in all plants examined (see Beevers, 1961; Beevers, Stiller and Butt, 1966). Figure 1 shows the series of interconversions occurring in the Krebs cycle.

The first reaction in the Krebs cycle involves the condensation of AcS-CoA and a component of the cycle, oxaloacetate, to form citrate. Citrate is further metabolized by the subsequent enzymes of the cycle. The only known way citrate can be synthesized is by the enzyme citrate synthetase. Degradation of citrate can be effected by several enzymes (Mahler and Cordes, 1966), namely aconitase, followed by isocitrate dehydrogenases, citrate lyase and citrate cleavage enzyme. The last two enzymes have not yet been found in citrus. Citrate synthetase was investigated in lemons (Srere and Senkin, 1966; Bogin and Wallace, 1966 b,c) and in grapefruit (Vines, 1968 a). The characteristics of both enzymes were similar, resembling the enzymes isolated from animal sources. Aconitase has not been investigated in citrus, but suggestions for its presence were obtained from numerous experiments. Isocitrate dehydrogenase exists in two distinct forms in citrus. One form, specific for NADP as electron acceptor, is present mainly in the cytoplasm (Buslig and Attaway, 1968 a). The other enzyme, present in the mitochondria (Buslig, unpublished), specifically uses NAD as electron acceptor. Other enzymes investigated in the Krebs cycle of citrus are the malate dehydrogenases (Vines, 1968 b), which are responsible for the generation of oxaloacetate which is necessary for the biosynthesis of citrate.

Fig. 1. The Krebs cycle.


Carbohydrates
pyruvate
CoASH
CO2
2[H]
acetyl CoA
Fatty acids
oxalacetate
citrate
cis-aconitate
threo-Ds-Ls-isocitrate
α-ketoglutarate
succinyl CoA
succinate
fumarate
Ls-malate
GTP
GDP
Pi
2H
H2O

In the experiments of Huffaker and Wallace (1959) the presence of PEP carboxylase and PEP-carboxykinase was also indicated. These enzymes also cause the formation of oxaloacetate, and may act as an acceleration mechanism for the formation of citric acid. The level of these enzymes was found higher in the more acid lemon fruits.

Examination of nicotinamide-adenine nucleotide redox ratios in citrus fruits (Bruemmer, 1969) showed an increase of the reduced form of the NADH/NAD ratio towards maturity, with little change of the NADPH/NADP ratio. This observation suggested that control of the activity of the Krebs cycle in citrus was located at the dehydrogenase levels, specifically at the malate dehydrogenase step.

An enzyme involved in the phosphorylation of adenosine nucleotides, adenylate kinase, which is related to respiratory energy metabolism, was also found in sweet and sour lemons. The level was significantly higher in the sour fruits (Abou-Zamzam and Wallace, 1970).

The major acid in citrus, citric acid, is formed, as mentioned earlier by the condensation of acetyl-CoA and oxaloacetate. This reaction is mediated by citrate synthetase which was found exclusively in the mitochondria in citrus (Srere and Senkin, 1966; Vines, 1968 a). The location has been found to be in the matrix in liver (Ernster and Kuylenstierna, 1970). Isocitrate dehydrogenase, as mentioned earlier, has two forms, the NADP- specific enzyme - mainly in the cytoplasm and the NAD - specific enzyme in the mitochondria. These enzymes directly control the level of citrate in the mitochondria. The activity of citrate synthetase is regulated by (1) the availability of acetyl-CoA, (2) the level of oxaloacetate, and (3) the level of ATP in the mitochondrion (Bogin and Wallace, 1966 c). The supply of pyruvate regulates the

availability of AcS-CoA. The evidence presented by Huffaker and Wallace (1959) indicates that pyruvate may also regulate the availability of oxaloacetate by the PEP carboxylation enzymes. Similar results were obtained by Bogin and co-workers (Bogin and Erickson, 1965; Bogin and Wallace, 1966 a) with pyruvate-3^{14}C and $^{14}CO_2$. The results of these experiments also gave some indication of the reversal of isocitrate dehydrogenase as a possible means of synthesizing citrate via isocitrate. Some of their experiments suggested the blockage of aconitase by citramalate as a way of accumulation of citric acids in lemons. Citramalate presumably would be formed by decarboxylation of parapyruvate utilizing hydrogenperoxide. In sour lemons the higher catalase activity would limit this reaction (Clark and Wallace, 1963). A possible in vivo regulation of citrate synthetase by ATP was examined in mature citrus fruit and a negative correlation was found between acidity levels and ATP concentration (Buslig and Attaway, 1969). However, the relation does not seem to hold true for younger fruit.

Since the enzymes of citric acid biosynthesis and immediate degradation (or reversal of such) are located in the mitochondria (Mahler and Cordes, 1966) it is obviously the primary site of synthesis. The amount of citric acid in the mitochondria is limited by the capacity of the organelle, along with the equilibrium of the Krebs cycle enzymes. Excess acids are probably transported through the mitochondrial membrane into the cytoplasm and subsequently into the vacuole (Beevers, et al., 1966). Spatial separation of the accumulated acids from the enzymes that would be acting on them predisposes for accumulation. The high H+ concentration in the vacuole is deleterious to most cytoplasmic constituents.

Information on the compartmentation in plant cells is available from data obtained with Kalanchoe leaves (McLennan, Beevers and Harley, 1963). From this system generalized conclusions may be drawn which are applicable to acid-accumulating plant organs, indicating the presence of metabolite pools for the cycle acids, distinct from mitochondria. A similar conclusion was reached in experiments with maize, where increasing vacuolation was accompanied by less availability of acids to enzymes in the cytoplams (or mitochondria). In this context it is important to note that factors controlling movement of solutes from mitochondria to cytoplasm to vacuole may be important in acid accumulation in plants. Ranson (1965) cites hypotheses involving active transport into the vacuole of specific acids that are overproduced or alternatively a genetically controlled system to again actively transport these acids into the vacuole. In citrus, where varieties of the same species are known to accumulate acids to different extents, the latter hypothesis is very attractive.

In commercial practice, some control of citrus acidity is possible by the use of lead arsenate in post-bloom sprays (Reitz, 1949), but the mechanism of action of this compound is quite obscure. The effective moiety is arsenate, which seems to cause more reduction of acids in wet seasons (Erickson, 1968). Vines and Oberbacher (1965) examined the effect of arsenate on mitochondria and suggested that the effect is due to uncoupling of respiration and phosphorylation. However, re-examination indicates the effect to be competition between arsenate and phosphate ions during respiration-linked phosphorylation.

Currently, investigation is directed towards the biochemical events occurring during growth of the citrus fruit. An understanding

of the biochemical events taking place during development, or more narrowly, citric acid accumulation, and changes in respiratory patterns, will give a better insight into the biochemical basis of acidity in citrus fruits.

MATERIALS AND METHODS

Plant Materials

Citrus fruits were obtained from the varieties listed in Table 1, from the Citrus Experiment Station collection (1, 2) and block plantings (3, 4), and the USDA Orlando Horticultural Station variety collection (5-9).

Sampling

Fruit of varying physiological ages was collected on the basis of size. In other cases collection was made in a chronological sequence or according to season (in mature fruit from the variety collection in Orlando). It was usually considered desirable to sample and compare materials from the same trees roughly in the same position to eliminate genetic and locational differences.

Preparation of tissue extracts

The fruits were homogenized by either Method A, outlined below, or by Method B. Both methods gave similar results with the substrates tested.

A. The weighed and peeled fruit was sectioned and grated using a stainless steel grater into an isolation medium containing 0.25M sucrose, 0.30M mannitol, 0.1M MOPS-KOH buffer, 0.05% BSA, 1% PVP-40, and 0.3% EDTA, adjusted to pH 7.5. During grating the pH was kept at 7.5 $\pm$ 0.3 and the temperature below 4° C.

B. The peeled and weighed fruit was solidly frozen by immersing in liquid nitrogen. After equilibration to the temperature of liquid

Table 1. Experimental materials

Type	Description	Pulp at maturity acidity range g/100g
1. Sour orange	Citrus aurantium L.	2.5-4.0
2. Bittersweet orange	C. aurantium var. Bittersweet	0.3-0.8
3. Marsh grapefruit	C. paradisi Macf.	0.9-1.5
4. Valencia orange	C. sinensis (L) Osbeck	0.5-1.2
5. Sampson tangelo	C. paradisi X C. reticulata	1.0-1.8
6. Wekiwa tangelo	C. paradisi X. Sampson	0.4-0.8
7. Baboon lemon	C. limon (L.) Burm.	3.5-4.5
8. Sweet lemon	C. limetta Risso	0.05-0.3
9. Succary acidless orange	C. sinensis	0.02-0.1

nitrogen the frozen tissue was broken into small pieces and ground to a fine powder in liquid nitrogen using the lowest possible speed in a pre-cooled, stainless steel Waring blender. The powdered pulp was left frozen in liquid nitrogen in a Dewar flask until added to isolation medium in 1:1 (w/v) proportion with constant stirring. The temperature and the pH were controlled at 0°C and 7.5 respectively, the latter by the addition of 20% KOH containing 0.3M mannitol.

With both methods, the resultant suspension was filtered through a nylon mesh (Nobel, 1967).

Isolation of mitochondria

Mitochondria were isolated from extracts as prepared above. The filtered suspension was centrifuged for 20 minutes at 15,000 x g. The supernatant was used to determine enzymic activities. The pellet was suspended in a wash medium, containing 0.25M sucrose, 0.3M mannitol and 0.1M MOPS-KOH at pH 7.5. Further fractionation was by differential centrifugation (Buslig and Attaway, 1968).

Measurement of mitochondrial respiration

Oxygen uptake of mitochondria was studied by either constant volume manometric techniques (Umbreit, Burris, and Stauffer, 1964), or polarographically using the Clark oxygen electrode (Yellow Springs Instruments).

The reaction mixture contained in a final 3 ml volume was 0.25M sucrose, 0.3M mannitol and 0.05M MOPS at pH 7.5: 100 μmoles substrate, 70 μmoles phosphate, 10 μmoles Mg^{2+}, 4 μmoles NAD, 0.1 μmole coenzyme A, 0.3 μmole thiamine pyrophosphate, 40 μg cytochrome c, 75 μg glucose, 400 μg hexokinase, and 5-10 mg mitochondrial protein. In case of the Warburg manometric measurements 15 μmoles ADP was added 30 minutes after the start of the experiment to measure the RCR. When respiration was measured by

the oxygen electrode, ADP was added to the mixture by a syringe after the initial respiratory rate stabilized.

Warburg manometry was performed at 30°C, oxygen electrode measurements at 25°C.

Determination of protein

Protein in mitochondrial extracts was determined with the Folin reagent (Sutherland, Cori, Haynes, and Olsen, 1948). The colorimetric determination was made by measuring the color intensity at 625mμ with a Bausch & Lomb Spectronic 20 spectrophotometer. Bovine serum albumin was used as standard.

Determination of phosphorylation

Phosphorylation was determined by measuring the glucose-6-phosphate formed with the glucose-hexokinase trap during the experiment by adding 0.5 ml of the reaction mixture to 4.5 ml 10% perchloric acid to stop the reaction. Supernatant (2.5 ml) was added to 1 ml 15% $KHCO_3$ solution. After removal of the precipitated $KClO_4$, 0.5 ml of this supernatant was added to 2.5 ml of the reaction mixture containing 20 units of glucose-6-phosphate dehydrogenase, 30 μmoles Mg^{2+}, 25 μmoles TRIS-HCl buffer (pH 7.8), and 50 μmoles NADP. The mixture was incubated at room temperature (25°C) for 15 minutes and absorbance was read against a reagent blank at 340 mμ in a Beckman DU spectrophotometer modified with a Gilford absorbance indicator.

Determination of enzymic activities

a) NADP-isocitrate dehydrogenase.

To 2.8 ml of reaction mixture containing 2.5 μmoles NADP, 12 μmoles isocitrate, 1.8 μmoles Mn^{2+}, and 25 mmoles TRIS-HCl buffer at pH 7.5, was added 0.2 ml of tissue extract (supernatant or sonicated, DMSO treated mitochondria).

b) NAD-isocitrate dehydrogenase.

To 2.8 ml of reaction mixture containing 2.5 μmoles NAD, 12 μmoles isocitrate, 1.8 μmoles Mn^{2+}, and 25 m moles TRIS-HCl buffer at pH 7.5 was added 0.2 ml of sonicated mitochondria, which has been diluted 1:1 (v/v) with DMSO.

c) Isocitrate lyase.

Measurement of isocitrate lyase was attempted by (1) measurement of the condensation of succinate and glyoxylate by determining the isocitrate formed with NADP-isocitrate dehydrogenase, (2) measurement of the forward reaction of glyoxylate reductase, (3) measurement of the forward reaction by colorimetrically determining the glyoxylate formed.

d) Citrate synthase.

Measurement of this enzyme was by the method described by Bogin and Wallace (1969).

Measurement of ATP and ADP concentrations

Concentration of ATP was measured by a modified firefly luciferin-luciferase system employing a Beckman liquid scintillation spectrometer (Buslig and Attaway, 1969). Each batch of firefly extract was standardized with known amounts of ATP. Concentration of ADP was measured as ATP after addition of creatine kinase (10 units) and creatine phosphate (10 mg) to 1 ml of the extract, followed by incubation at room temperature for 30 min. After the determination of the total ATP in this preparation the concentration of ADP was calculated as ADP = ATP total - ATP. In every case determination of ADP was followed by ATP to obtain the results.

Measurement of whole fruit respiration

Carbon dioxide of whole fruit and leaves was measured by a Beckman infrared CO_2 analyzer. The fruit or leaves were enclosed in containers

equipped with continuous air flow, maintaining approximately 3 inches of head pressure. The analyzer was connected to the exit port of a series of solenoids which permitted sequential monitoring of 12 containers (20 minutes each). At the end of the 20 minute period a multipoint recorder indicated on a chart recorder the CO_2 concentration of the vessel measured.

Acidity measurements

Titratable acidity was determined by volumetric measurement, employing standardized KOH solutions.

Incorporation of $^{14}CO_2$ into whole peeled citrus fruit

The fruits to be tested were carefully peeled and placed in beakers. The beakers were placed in a 4 liter container that could be tightly sealed. For $^{14}CO_2$ generation 0.8 mg of barium carbonate (^{14}C) (52.2 μC/ μmole) was also placed in the chamber and 5 ml 2M H_2SO_4 was added through a port after sealing. The fruit was allowed to remain in this atmosphere in the dark for 4 hrs. At the termination of the exposure to $^{14}CO_2$ the jar was flushed for 5 min with compressed air, the exiting gases scrubbed with 5M KOH to trap all remaining $^{14}CO_2$. Juice was expressed from the pulp by hand, followed by counting of the radioactivity of a small portion of the juice.

An aliquot of the juice was fractionated into neutral, anionic and cationic fractions by chromatography on Dowex-50(H^+) and Dowex-1(OH^-) columns. The effluents were analyzed for radioactivity by scintillation counting.

Radioactivity measurements

Radioactive $^{14}CO_2$, evolved during tracer experiments with Warburg manometry, was collected in the center well by absorbing into 6M KOH

solution adsorbed onto fluted filter paper. Counting was done in 15 ml toluene containing 0.475% PPO and 0.025% dimethyl-POPOP (Attaway and Buslig, 1968) by dropping the piece of filter paper in the solution and using an ambient Beckman liquid scintillation spectrometer.

Aqueous solutions were counted by adding 0.5 ml BBS-2 solubilizer (Beckman Instruments) to the counting solution.

RESULTS

Titratable acidity and the respiratory activity of various citrus fruits

Two distinct patterns of organic acid accumulation were observed during the growth of the citrus fruits studied. These patterns are represented by sour lemon, sour orange, and bittersweet orange, and are shown in Fig. 2. The essential difference, besides the levels of organic acids accumulated, are in the general characteristics of these curves. Lemon, which accumulates the highest amount of acid, follows a continuously increasing line throughout the growth of the fruit. The curve is steepest at the initial stages of the growth cycle, slows down about 1/3 full size and continues at a linear rate for the rest of the period. Sour oranges show an even higher initial rate of accumulation, reaching a maximum at approximately 1/3 full size, tend to stay at a plateau for a short period, followed by a slow decline as the fruit increases in size. Bittersweet oranges accumulate very low concentrations of organic acids, with an extended plateau, similar to sour orange, declining toward the end of the growth of the fruit. In all three cases the predominant organic acid is citric acid.

Respiration of the whole fruits show no particular differences with the exception of the apparent initial rate at the beginning of the growth phase. The initial respiratory rate of the sweeter variety shows a higher level of activity than the sour fruits. Figure 3 shows the

Fig. 2. Patterns of titratable acid accumulation in bittersweet and sour oranges and in sour lemon. Arrows indicate mature fruit stage.

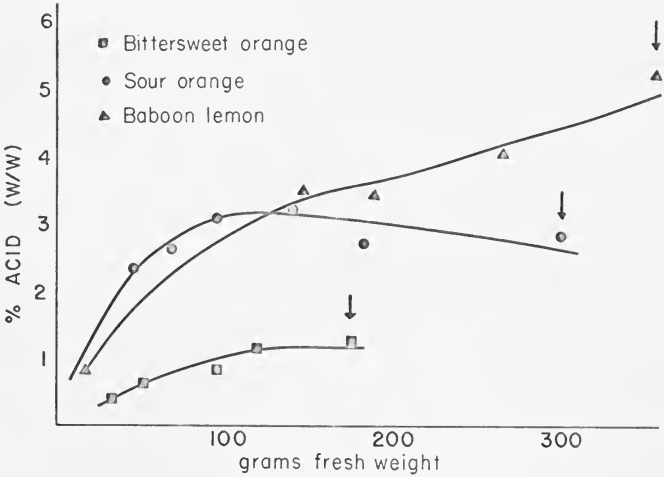
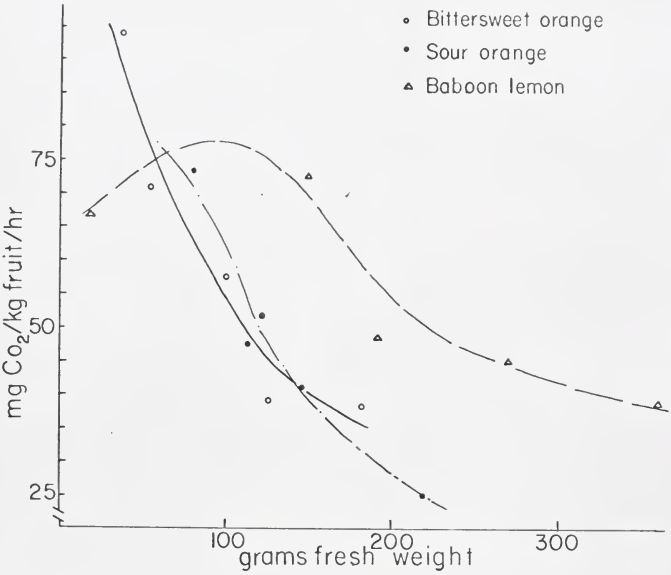

Bittersweet orange
Sour orange
Baboon lemon
% ACID (W/W)
1
2
3
4
5
6
100
200
300
grams fresh weight

Fig. 2. Patterns of titratable acid accumulation in bittersweet and sour oranges and in sour lemon. Arrows indicate mature fruit stage.


Bittersweet orange
Sour orange
Baboon lemon
75
50
25
mg Co_2/kg fruit/hr
100
200
300
grams fresh weight

respiratory activity of the three types examined during their respective growth cycles. The general pattern shows a continuous decline until maturity. Examination of mature fruits of various acidity levels indicate no drastic differences in their respiration. Table 2 shows a comparison of titratable acidity and rate of respiration between the fruits examined.

Respiratory activity of isolated fruit mitochondria

Mitochondria isolated from various types of citrus fruits at maturity showed a considerable variation in their ability to metabolize a series of substrates. Their response to exogenous ADP also showed a wide range of variation. Generally, it was concluded that the activity of sour fruits was inferior to that of the sweet fruits. The lower respiratory control ratio (RCR), the ratio of respiratory activity in the presence of exogenous ADP vs basal respiratory activity in the mitochondria, is also an indication of such a difference. The results obtained from mature fruit are shown in Table 3.

During fruit development respiratory activity of sour and bittersweet orange mitochondria was followed. The results indicate a generally lower level of respiration of the sour variety with all substrates tested. Response to added ADP, the RCR, was consistently lower with mitochondria isolated from the sour fruits at any point of the growth period. Figures 4 and 5 indicate respiratory activity of bittersweet and sour orange mitochondria, respectively. Figure 6 shows the response endogenous respiration to ADP. The most notable feature of the respiratory activity of bittersweet orange mitochondria during the period of observation was the apparent synchrony of activity with every substrate employed. In contrast the sour orange mitochondria exhibited a biphasic

Table 2. Acidity and respiration of mature citrus fruits

Type	% Titratable acid/pulp	Respiration mg CO_2/kg/hr
Bittersweet orange	0.57	38.50
Sour orange	2.78	25.16
Sampson tangelo	1.95	38.61
Wekiwa tangelo	0.53	41.09
Succary acidless orange	0.09	38.23
Orange de Nice	1.12	34.37
Sweet lemon	0.11	27.91
Baboon lemon	5.24	39.45

Table 3. Respiration of mature citrus fruit mitochondria*

	RCR	O_2 uptake	P/O	RCR	O_2 uptake	P/O	RCR	O_2 uptake	P/O
Substrate	Bittersweet orange			Sour orange			Wekiwa tangelo		
Succinate	1.37	4.48	1.58	1.00	1.28	0.11	1.14	4.05	0.78
Citrate	2.26	1.58	2.09	0.54	-0.12	-	5.12	2.04	1.24
α-Ketoglutarate	2.20	4.74	2.40	3.00	0.60	0.47	1.53	2.85	1.74
Pyruvate+malate	1.83	4.32	2.68	0.11	0.08	0	2.14	2.89	1.11
	Sampson tangelo			Baboon lemon			Sweet lemon		
Succinate	1.53	2.81	0.26	0.26	0.09	0	1.42	3.90	1.11
Citrate	5.82	1.22	0.34	0.26	0.08	-	1.77	1.38	1.87
α-Ketoglutarate	1.23	2.40	0.87	-	-	-	1.87	2.67	2.28
Pyruvate+malate	1.75	2.58	0.43	0.01	0	0	0.90	1.59	3.32
	Valencia orange			Acidless orange			Marsh grapefruit		
Succinate	-	7.20	1.92	2.17	3.16	1.48	1.32	2.77	1.29
Citrate	-	3.94	4.00	1.40	0.66	1.08	9.33	0.74	2.12
α-Ketoglutarate	-	6.62	3.00	1.55	2.87	2.19	2.18	1.73	2.58
Pyruvate+malate	-	3.80	3.88	1.71	1.54	2.07	1.26	1.40	2.49

*O_2 uptake is μatoms/hr/mg mitochondrial protein.

Fig. 4. Respiration of bittersweet orange mitochondria.

endogenous
citrate
succinate
α-ketoglutarate
pyruvate + malate
μ atoms O2/mg mitochondrial protein/hr
5
100
200
300
grams fresh weight

Fig. 5. Respiration of sour orange mitochondria.

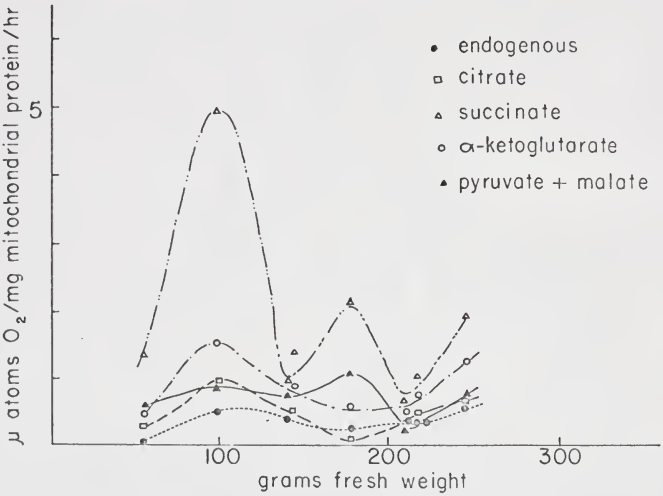
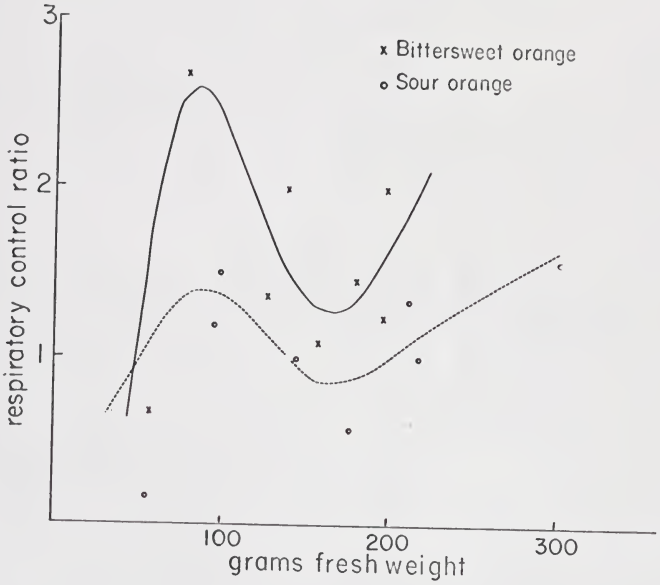

μ atoms O_2/mg mitochondrial protein/hr
5
100
200
300
grams fresh weight
endogenous
citrate
succinate
α-ketoglutarate
pyruvate + malate

Fig. 6. Respiratory control ratio changes during development.


3
2
1
respiratory control ratio
x Bittersweet orange
o Sour orange
100
200
300
grams fresh weight

type of activity with all substrates showing synchrony at the beginning of the growth curve, succinate and pyruvate + malate showing a second smaller increase in activity while the rest of the curves hit minima. The RCR (Fig. 6) curves of endogenous respiration in both instances show similar shapes, but the bittersweet orange mitochondria indicate a considerably higher respiratory control by ADP at all comparable points.

Enzymic activities of fruit pulp during growth

For evaluation of some of the enzymes contributing to the synthesis and to the degradation of citric acid, which is the predominant acid in these fruits, the enzymes citrate synthetase, 2 isocitrate dehydrogenases, NADP and NAD specific, and isocitrate lyase were selected.

Citrate synthetase activity is shown in Figures 7 and 8 for bittersweet and sour oranges, respectively. The curves indicate a comparable initial activity in both fruit types, with bittersweet orange having a slightly higher initial activity. As the fruits grew, both fruits showed an increase in the enzymic activity, followed by a decline and an apparent plateau for the bittersweet orange. The sour orange shows a slight decrease in activity, followed by another increase in activity. Total enzyme per fruit increases with both varieties, however the final level was considerably higher in the sour fruit.

Isocitrate dehydrogenase (NADP-specific) showed a somewhat different pattern. In the sour fruit, there is an initial rapid decline, reappearance and another decline, ending in a constant level with fruit from about 2/3 full size to maturity. The bittersweet orange shows an initial plateau, followed by a rapid decline at approximately 1/2 full size, ending in a slow decrease toward maturity. Figures 9 and 10 show

Fig. 7. Citrate synthetase activity of bittersweet oranges.

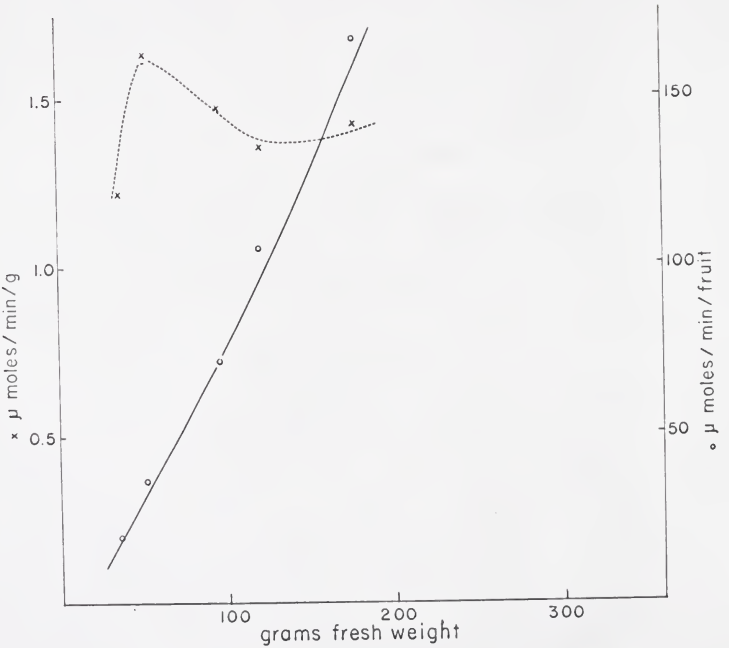

1.5
1.0
0.5
× μ moles / min / g
150
100
50
∘ μ moles / min / fruit
100
200
300
grams fresh weight

Fig. 8. Citrate synthetase activity of sour oranges.

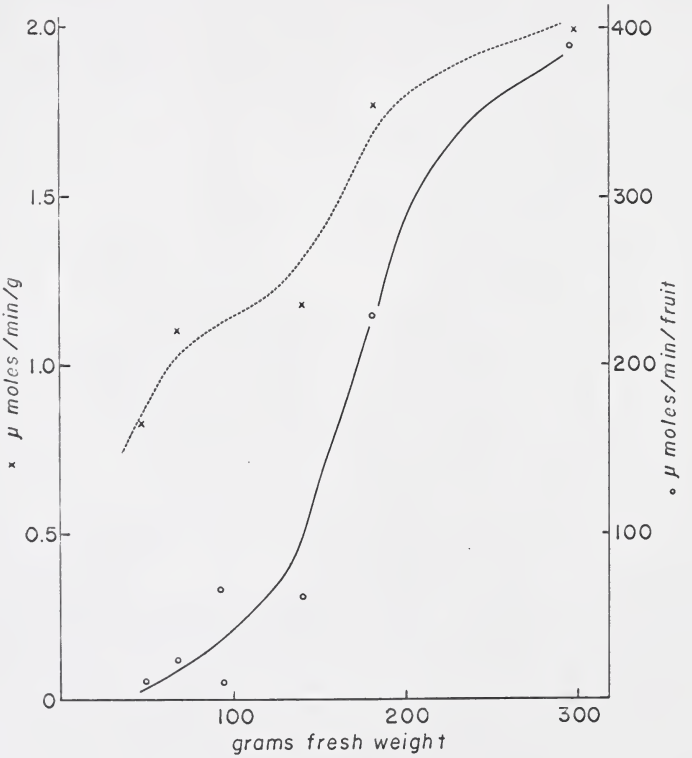

2.0
1.5
1.0
0.5
0
× μ moles/min/g
400
300
200
100
• μ moles/min/fruit
100
200
300
grams fresh weight

Fig. 9. NADP-isocitrate dehydrogenase activity of bittersweet oranges.

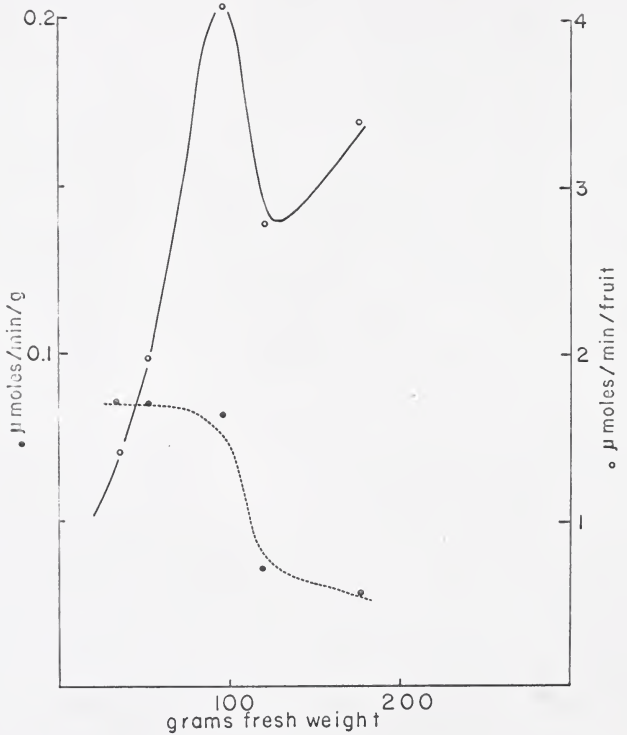

0.2
0.1
4
3
2
1
• μ moles/min/g
∘ μ moles/min/fruit
100
200
grams fresh weight

Fig. 10. NADP-isocitrate dehydrogenase activity of sour oranges.

0.15
0.10
0.05
μ moles/min/g
300
200
100
μ moles/min/fruit
100
200
300
grams fresh weight

rates of activity and total activity per fruit. The total activity curves are very similar in both fruit types, although bittersweet seems to have higher activity over all.

Isocitrate dehydrogenase (NAD-specific) activity seems to be quite similar in both types of fruit. The levels of activity are comparable. Figures 11 and 12 show rate and total activity.

Isocitrate lyase was not found in any of the citrus fruits examined thus far.

Concentrations of ATP and ADP in fruit pulp

The concentration of ATP and ADP was measured in the three types of fruit with the organic acid accumulative patterns described earlier. The results are shown in Table 4. The most notable information that can be obtained from these figures is the initially higher level of both nucleotides in the pulp of the sour lemon fruit, lower in the sour orange, and finally lowest in the bittersweet orange. At the end of the growth period, the order is reversed, with bittersweet showing the highest observed concentration of both.

Metabolism of ^{14}C-citrate and ^{14}C-pyruvate by isolated mitochondria and whole juice sacs

In these experiments the mitochondria were isolated from mature citrus fruits. The results are shown in Table 5. It may be noted that as before respiratory efficiency of the bittersweet orange mitochondria is greater than that of the sour orange. These results are in agreement with earlier observations, shown in Figures 4 and 5, for comparable stages of fruit growth.

Table 6 lists the results obtained with whole, isolated juice sacs. These results once again reconfirm the higher efficiency of the less acid fruit to metabolize Krebs cycle intermediates.

Fig. 11. NAD-isocitrate dehydrogenase activity of bittersweet oranges.

15
10
5
• μmoles/min/g
600
400
200
° μmoles/min/fruit
100
200
300
grams fresh weight

Fig. 12. NAD-isocitrate dehydrogenase activity of sour oranges.

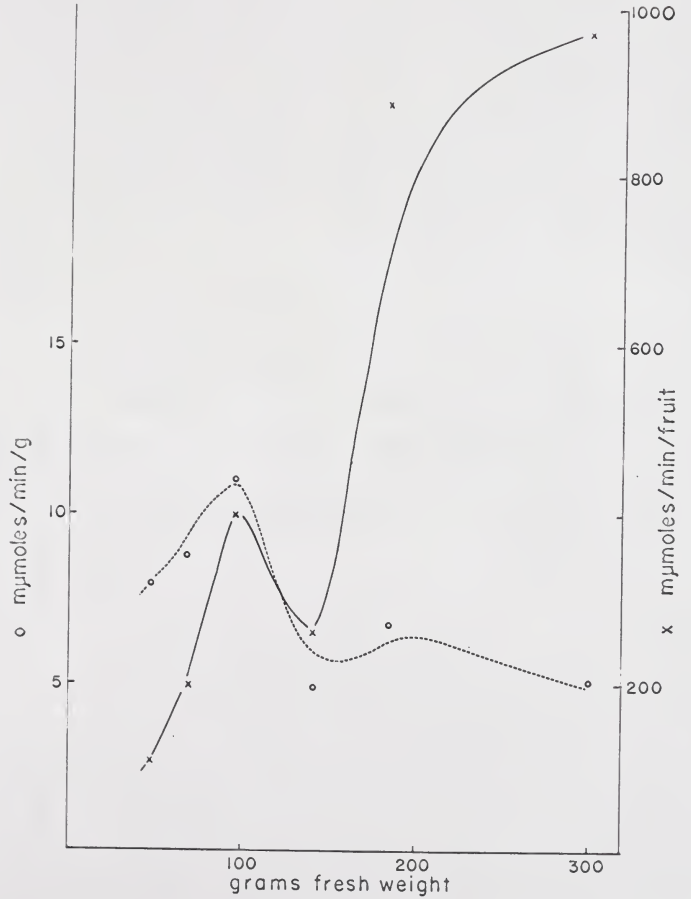

o mμmoles/min/g
x mμmoles/min/fruit
15
10
5
1000
800
600
200
100
200
300
grams fresh weight

Table 4. Concentration of ATP and ADP during development in Baboon lemon, bittersweet and sour oranges

Average weight per fruit (g)	ATP m μ moles/g pulp	ADP m μmoles/g pulp	ATP ADP
	Baboon lemon		
17.95	143.6	180.5	0.78
147.00	48.9	35.2	1.39
189.25	53.6	35.9	1.49
266.00	38.4	44.3	0.87
357.05	37.6	31.8	1.18
	Bittersweet orange		
35.05	62.8	130.9	0.48
52.17	48.0	56.1	0.86
96.00	56.2	32.5	1.73
120.10	37.0	33.2	1.12
176.25	38.9	39.9	0.98
	Sour orange		
18.40	121.2	113.1	1.07
37.60	110.8	44.2	2.50
55.00	64.6	60.7	1.06
137.00	40.0	54.2	0.74
224.00	30.3	16.1	1.88

Table 5. Metabolism of radioactive citrate and pyruvate by isolated mitochondria

Substrate	Sampson tangelo		Wekiwa tangelo	
	O_2 uptake μatoms*	$^{14}CO_2$ released cpm*	O_2 uptake μatoms*	$^{14}CO_2$ released cpm*
Citrate-^{14}C(1,5) -ADP	0.17	33	0.40	34
Citrate-^{14}C(1,5) +ADP	1.22	127	2.04	233
Pyruvate-^{14}C(2) +malate-ADP	1.47	26	1.35	27
Pyruvate-^{14}C(2) +malate+ADP	2.58	30	2.89	34

*Both are per hr/mg mitochondrial protein

Table 6. Metabolic activity of whole juice sacs

Substrate	Bittersweet orange		Sour orange	
	O_2 uptake μatoms*	$^{14}CO_2$ released cpm*	O_2 uptake μatoms*	$^{14}CO_2$ released cpm*
Endogenous	1.83	-	1.65	-
Citrate-^{14}C	2.03	2.84	1.47	2.73
Pyruvate-^{14}C	2.82	0.64	1.49	0.61

*Both are per hr/g juice vesicles

Dark fixation of $^{14}CO_2$ by whole peeled fruit

The fixation of $^{14}CO_2$ by peeled bittersweet and sour oranges was examined. The results indicate a lower extent of incorporation of radioactivity into the juice of sour oranges. In Table 7 the level of incorporation is indicated, with the results obtained on fractionation of the extracted juice into neutral, cationic and anionic fractions. It is assumed that these fractions represent largely sugars, amino acids and organic acids, respectively. The sour orange seems to be able to incorporate a greater proportion of dark fixed carbon dioxide into the neutral fraction, and a lower proportion into the amino acid fraction than bittersweet. Incorporation into organic acids seems to be to approximately the same level, although due to the differential in acidity levels between the two, a greater percentage of the acids in bittersweet oranges are labeled.

The effect of arsenate on mitochondrial activity of Valencia oranges

For this experiment Valencia oranges were selected instead of the commercially sprayed grapefruit, due to the greater sensitivity of these fruits to the effects of arsenate. In Table 8 the results of these experiments are shown. It is apparent from the results obtained that a concerted reduction in respiratory associated activity occurs, as both oxygen uptake and phosphorylation were decreased. The decrease in oxygen uptake seems to be proportional to the decrease in phosphorylation, rather than causing uncoupling attributed to arsenate.

Table 7. Incorporation of $^{14}CO_2$ into whole peeled fruit*

	Total ^{14}C incorporated/ml juice cpm	^{14}C incorporated cpm/ml		
		Neutral	Cationic	Anionic
Bittersweet orange	23815	9535	1570	6625
Sour orange	20552	11856	653	6403

*Results are expressed as incorporation/4 hrs

Table 8. Respiration of Valencia orange mitochondria*

Substrate -AsO_4	O_2 uptake µatoms/hr	P_i esterified µmoles/hr	P/O	P/O** corrected for endogenous
Endogenous	0.41	1.33	3.24	-
Succinate	6.61	5.18	0.78	0.62
Citrate	2.38	3.56	1.50	1.13
α-Ketoglutarate	5.59	6.41	1.15	0.98
Pyruvate + malate	4.05	5.27	1.30	1.08
Substrate + 10^{-2}M AsO_4				
Endogenous	0.47	1.34	2.85	-
Succinate	6.03	3.98	0.66	0.48
Citrate	2.12	2.46	1.16	0.68
α-Ketoglutarate	2.44	3.39	1.39	1.04
Pyruvate + malate	2.46	3.16	1.29	0.91

*Results expressed are per mg mitochondrial protein

**These results represent activity of mitochondria above basal rate

DISCUSSION

The experiments were designed to investigate the problem of citric acid accumulation in citrus fruits. This phenomenon involves several facets of fruit metabolism, some of which are incidental and some are integral parts of the organic acid accumulation process.

The early work on organic acid accumulation in citrus (Bartholomew, 1923) was concerned mainly with the patterns of increase of titratable acids. Later workers (Sekhara Varma and Ramakrishnan, 1956; Sinclair and Ramsey, 1944; Harding and Fisher, 1945; Bain, 1958; Ting and Deszyck, 1959; Clements, 1964 a, b; Rasmussen, 1964; Ting and Vines, 1966) examined the quantitative distribution of some of the organic acids found in the fruits. From such investigations it became apparent that citric acid was, in most instances, the predominant organic acid in citrus (Erickson, 1968). Biosynthetic pathways leading to the formation of citric acid are well known since the pioneering work of Krebs and Johnson (1937), who demonstrated this acid as the integral part of aerobic respiratory pathways in tissue homogenates. Now it seems reasonable to conclude that all living organisms form this compound to varying extents.

Since organic acid metabolism and respiration are very closely connected phenomena, the sequence of experiments is purported to reflect a series of observations, starting on the outside of the fruit with gross respiratory patterns, along with measurement of titratable acidity during growth (Figures 2 and 3, Table 2). Metabolic activity that can be concluded from these results indicates the reduction of respiration as

the fruit grows, along with the reduction of surface/volume ratio, and in all cases, the increase of acidity. Since the results of respiration do not parallel the accumulation pattern, further examination on the subcellular level, such as isolated mitochondrial respiration, gives a considerably more accurate indication of respiratory activity as linked to acidity. Considering the various citrus fruits (Table 3), each with different acidity characteristics, it may be safely concluded that a correlation exists between acidity level and the respiratory activity of isolated mitochondria. Seasonal variations are reflected in examination of RCR of bittersweet and sour oranges (Fig. 6) and respiratory activity of both types of mitochondria, respiring various substrates (Fig. 4, 5). The apparent synchrony of the metabolism of substrates by bittersweet orange mitochondria and the asynchrony observed with sour orange mitochondria, along with the efficiency of metabolism of these substrates is indicative of some differences in the controlling mechanism of respiration. It is readily apparent, that citrate is metabolized by sour varieties at a lower rate than the "normal", not quite so acid, varieties. In fact respiration of sour orange mitochondria show a tendency to be inhibited by the substrate concentration used below the endogenous level in the presence of citrate. It is also interesting to note, that succinate and pyruvate + malate (Fig. 5), which all precede citrate in the normal sequence of respiration, are the efficient substrates at this stage of growth while citrate, and subsequently α-ketoglutarate, are poorly metabolized at the same time. This could result in the accumulation of citrate.

Confirmation of the higher efficiency of less acid varieties was obtained by metabolizing ^{14}C-pyruvate, and ^{14}C-citrate both by isolated

mitochondria alone, and also by whole juice sacs (Tables 5, 6). In these experiments the juice sacs showed unexpectedly higher rates of equilibration between exogenous substrate and the cell endogenous reserves with sour orange, while both substrates caused some depression of the respiratory rate. With juice sacs from bittersweet orange, this equilibration was considerably slower, resulting in an O_2/CO_2 ratio of 0.36 with citrate and 2.20 with pyruvate versus 0.27 and 1.22 respectively for sour orange.

The higher efficiency of metabolism of a less acid variety is especially significant when further results are examined (Table 5). These two tangelos are very closely related. Sampson was the maternal parent of Wekiwa, but they are quite distinct as far as their acidity levels (Table 1). It is very convincingly shown that the respiratory activity of these mitochondria is very efficiently controlled in Wekiwa by exogenous ADP, but not nearly as well in Sampson. With citrate as substrate, Wekiwa mitochondria respire and degrade citrate about twice as fast as Sampson mitochondria, while the absolute level of pyruvate metabolism changes very little, indicating some possible control of pyruvate entry into the mitochondria when applied exogenously. This result is not very clearly explained at this time.

The enzymic reactions involved in the biosynthetic and degradative pathways of citrate were the first objects of investigation in citrus fruits. Initial observation by Sekhara Varma and Ramakrishnan (1956) suggested that citrus fruits are capable of synthesizing the organic acids found in them. Erickson (1957) came to a similar conclusion through grafting sour lemon on sweet lemon stock and the reverse, without significantly altering organic acid composition of the fruit. A

series of articles from California (Huffaker and Wallace, 1959; Clark and Wallace, 1963; Bogin and Erickson, 1965; Bogin and Wallace, 1966 b) indicated the involvement of metabolic blockage by an inhibitor, which seemed to form at a side reaction, along with the reversal of some of the Krebs cycle reactions, as the cause of the accumulation of citric acid. The reactions that were involved, individually at least, are capable of the synthesis of citric acid or an immediate precursor.

Enzyme activities involved in the synthesis and degradation of citric acid are shown with increasing size (maturity) of bittersweet and sour orange fruits (Figs. 7 to 12).

Citrate synthetase activity of bittersweet orange (Fig. 7) seems to be quite high at the beginning stages of growth, reaches a climax at about 1/4 maximum size, declines slowly to a plateau about 1.3-1.4 μmoles/min/g pulp. Total activity present in the pulp increases nearly linearly until maturity. Sour oranges present an entirely different citrate synthetase activity pattern (Fig. 8). The initial activity is lower than in the bittersweet orange, but it increases rapidly, slows somewhat at 1/2 full fruit size, and again shows a very rapid increase. It seems to reach a plateau perhaps at maturity, considerably higher than bittersweet orange. Total activity increases rapidly after an initial lag, slowing at maturity, so as to present a sigmoid-shaped curve. The difference in activity during growth is a good indication of a higher metabolic control of this enzyme during development.

The two enzymes intimately involved in the degradative pathway of citrate follow aconitase, which establishes a ready equilibrium between citrate, cis-aconitate and isocitrate. Isocitrate degradation is readily accomplished by either NADP or NAD specific isocitrate

dehydrogenases. Both are present in citrus (Buslig and Attaway, 1968 a; Buslig, unpublished). Activity of the NADP-specific isocitrate dehydrogenase in bittersweet orange shows a higher initial activity which slowly declines until the mid-point of growth, drops very rapidly to 1/3 initial level and declines further slowly until maturity. Sour orange shows a rapid drop initially, followed by a transient rise, falling back to a level about equivalent to the first low point. Total activity in both instances seems to follow the same general pattern, although higher activity can be observed with bittersweet throughout the growth cycle (Figs. 9, 10).

NAD-isocitrate dehydrogenase activities are quite similar in both fruits, differing towards maturity as sour orange NAD-isocitrate dehydrogenase declined while bittersweet orange actually showed an increase (Figs. 11 and 12).

It is interesting to observe that bittersweet orange showed increasing activity of the degradative isocitrate dehydrogenases after citrate synthetase reached its peak, while sour oranges show an initially higher activity of these enzymes, but both decline as citrate synthetase continues to increase.

These results indicate that the activity of citrate synthetase alone may be sufficient to account for the rise in acidity in sour oranges, while the closely related bittersweet variety seems to have control of not only the general respiratory pattern during growth but citrate synthetase as well.

Mitochondrial asynchrony of metabolism was observed in sour orange, which along with the enzyme activity patterns could account for the observed accumulation pattern of citric acid. These results are in

agreement with previous investigators (Erickson, 1958) who observed similar patterns of accumulation. However, the present information on enzymic patterns would seem to indicate that at least part of the accumulation of citrate could be due to activities of citrate synthetase and isocitrate dehydrogenase levels without a complex inhibitory system as they described.

Huffaker and Wallace (1959) and Clark and Wallace (1963) indicated in their papers that reversal of some decarboxylation reactions may account for accumulation of citrate. Dark fixation of $^{14}CO_2$ by the two types of oranges (Table 7) is in agreement with their results; however it is obvious that such levels of incorporation, especially the closeness of the results, will not account for the great differences in acidity observed.

In earlier work with citrus no attempt was made to explain the accumulation of citric acid to a level, which if present in the mitochondria or cytoplasm without some type of barrier, that would be quite deleterious to the entire fruit. Recently Abou-Zamzam and Wallace (1970) examined adenylate kinase, one of the enzymes which is possibly involved in energy balance within the cell. Such a reaction could shed light on the problem of accumulation, as it may be able to account for the energy requirements to remove the high concentration of citric acid from vulnerable areas to the vacuole by active transport. Adenosine nucleotides, especially ATP, were found to be higher during growth in sour orange and baboon (sour) lemon than in bittersweet orange (Table 4). In lemons a similar situation exists (Abou-Zamzam, Wallace and Motoyama, 1970). As organic acids accumulate, ATP levels decline in all cases. ATP/ADP

ratios were found quite variable, generally increasing during the greatest rate of increase in acidity. Hydrolysis of ATP may be able to supply the needed energy for translocation of the acids into the vacuole, where it may be accumulated without deleterious effects. Such compartmentation would require additional energy for maintenance of high H^+ concentrations in the vacuole. Assuming a concentration gradient of 4 (pH within vacuole 3.5, in cytoplasm 7.5), energy required to establish this gradient would be (at 20°C):

$$G^\circ = 2.303\ RT \log \frac{10^{-3.5}}{10^{-7.5}}$$

$$G^\circ = 2.303 \times 1.98 \times 293 \times 4 = 5344 \text{ cal/mole}$$
$$5344/8000 = 0.67 \text{ moles ATP}$$

hydrolized to establish the gradient (Lehninger, 1965). Of course the higher the accumulation, the higher the requirement.

Compartmentation and active transport would agree very well with the present results. It is obvious that genetically controlled enzyme systems operate in citrus, especially on the basis of closely related varieties. The genetically controlled active transport system (Ranson, 1965) and evidence presented by Oaks and Bidwell (1970) would also be supported by these findings.

In citrus fruit some control of acidity can be achieved by the application of post-bloom sprays of lead arsenate (Reitz, 1949). It was suggested, that the mechanism of action of this compound is that of an uncoupler, removing oxidative control of phosphorylation in the mitochondrial (Vines and Oberbacher, 1965). Repetition of these experiments indicated a minimal degree of uncoupling (Table 8). The results pointed

to competition of arsenate with phosphate, as about an equal degree of reduction was obtained in both oxygen uptake and phosphorylation.

In conclusion, the present results indicate the control of citric acid accumulation to lie in part in the control of the enzymes citrate synthetase and the subsequent degradative dehydrogenases. Adenosine nucleotides play a controlling influence on the concerted respiratory mechanism of the Krebs cycle, with a possible role in the transport mechanism from the mitochondria and cytoplasm to the vacuole. It does not seem likely that dark CO_2 fixation plays a major role in acid accumulation.

SUMMARY

Respiratory and metabolic interrelationships involving biosynthesis and degradation of citric acid in citrus fruits were studied. The data obtained indicate the following:

1. Citrate accumulation in part is due to altered control of citrate synthetase in the more sour variety.

2. Energetics of acid accumulation may be accounted for by the higher levels of ATP and higher ATP/ADP ratios in sour fruits during the greatest increase in citric acid content.

3. Asynchrony in favor of citrate production exists with the enzymes examined in the sour fruit, while the sweeter variety shows a reasonable synchrony.

4. Control of respiration and of Krebs cycle activity by exogenously applied ADP in the mitochondria in citrus fruits was more efficient in the sweeter variety.

5. Variations between the sweet and sour varieties seem to be due to a primary genetic control of the enzymes involved.

6. The action of arsenate on respiration in citrus fruits seems to be due to competition with phosphate. In contrast earlier interpretation considered it to be an uncoupler of oxidative phosphorylation.

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BIOGRAPHICAL SKETCH

Béla Stephen Buslig was born on August 27, 1938 in Budapest, Hungary. He attended primary and secondary schools there. In 1956, following the Revolution, he escaped to Austria. Subsequently he lived in England and Canada, where he completed his undergraduate training at Queen's University at Kingston, Ontario, receiving a degree of Bachelor of Arts (General) in 1962 with concentration in biology and chemistry. Following graduation he attended Western Reserve University as a N.I.H. research fellow in microbiology. In 1963 he enrolled in the Florida State University Graduate School pursuing a program of study in genetics. In 1967 he obtained the degree of Master of Science in biology, with emphasis on the molecular genetics of bacteria. Soon after graduation he started working as a chemist for the Florida Citrus Commission at the Lake Alfred Citrus Experiment Station. At the same time he proceeded with further studies in the Department of Fruit Crops at the University of Florida, to obtain the degree of Doctor of Philosophy, concentrating on fruit physiology.

He is a member of the Canadian Society of Plant Physiologists, American Chemical Society, Genetics Society of America, American Association for the Advancement of Science, New York Academy of Sciences, Florida State Horticultural Society and the American Society for Horticultural Science.

He is married to the former Bertha Horsfall and has two children. Presently he is employed by the Scientific Research Division of the Florida Department of Citrus as a Research Biochemist.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert H. Biggs, Chairman
Professor (Biochemist), Fruit Crops

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John A. Attaway, Co-chairman
Professor (Chemist), Citrus Experiment Station

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Alfred H. Krezdorn
Professor (Horticulturist), Fruit Crops

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William F. Newhall
Professor (Biochemist), Citrus Experiment Station

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David S. Anthony
Professor (Biochemistry), Botany

This dissertation was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1970

Dean, College of Agriculture

Dean, Graduate School